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TRANSPORT AND PHOSPHORYLATION OF D-GALACTOSE IN RENAL CORTICAL CELLS

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SUMMARY

An improved analytical procedure for the extraction and determination of total, free and phosphorylated tissue sugar is described. This method, employing $ZnSO_4$ plus $Ba(OH)_2$ for the precipitation of sugar phosphates, yields values identical with those obtained by the more laborious separation of free and phosphorylated sugar by ion-exchange chromatography. Erroneous values for free sugar due to the action of a Zn^{2+}-activated phosphatase and/or the lability to acids of some sugar phosphates, are avoided.

Using this technique for the study of transport and phosphorylation of D-galactose in rabbit renal cortical slices and tissue extracts, it was found:

1. The cellular uptake of D-galactose was associated with the appearance of both free and phosphorylated sugar whether or not external Na^+ was present. At 1 mM sugar, galactose was accumulated in the cells against a modest concentration gradient of 1.445 ± 0.097 ($n = 17$). Galactose phosphate appeared in the cells considerably faster than free sugar under conditions of net uptake as well as of steady-state exchange (pulse-labelling).

2. Increasing saline pH (6–8) increased the cellular levels of sugar phosphate without affecting the steady-state values of free sugar. With tissue extracts, increasing pH also stimulated the activity of galactokinase and the dephosphorylation of galactose 1-phosphate by a Zn^{2+}-activated phosphatase.

3. 0.5 mM phlorizin inhibited the tissue uptake of galactose and its subsequent oxidation to CO_2 only to a minor degree (30 and 10 %, respectively). The absence of external Na^+ further depressed the phlorizin effect. Preincubation of the tissue with phlorizin and subsequent washing in part abolished the inhibitory effect. The data suggest that a major portion of the galactose uptake by the tissue proceeds by a mechanism with a low affinity for phlorizin.

4. Efflux studies showed that the wash-out of free galactose from slices was associated with a net decrease of both free and phosphorylated tissue sugar.

5. The above results suggest the possibility that phosphorylation may represent a step in the Na^+-independent, phloretin-sensitive transfer of D-galactose across the

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Abbreviations: Gal, D-galactose; 2-d Gal, 2-deoxy-D-galactose; d Gal-1-*P*, D-galactose-1-phosphate; Glc, D-glucose; 2-d Glc, 2-deoxy-D-glucose; TES, *N*-tris(hydroxymethyl)methylaminoethane sulphonic acid.

antiluminal cell membrane. The participation of intracellular galactokinase and a Zn^{2+}-activated alkaline phosphatase in the maintenance of the steady state of free and phosphorylated galactose in the cells has been demonstrated.

INTRODUCTION

This study was prompted by several observations. It was found that using rabbit renal cortical slices, variations of external pH greatly affected the rates of active transport as well as the steady-state levels of sugars such as D-galactose, 2-deoxy-D-glucose and 2-deoxy-D-galactose [1]. Since evidence was available to show that at least the first two sugars are metabolized by renal cortical cells [2–5] the question had to be considered whether the pH effect on the transport might be related to the actual transport step, or to the intracellular metabolism of these sugars. The latter interpretation would be consistent with the fact that in rabbit kidney the cellular accumulation of methyl-α-D-glucoside i.e. a sugar metabolized, if at all to only a minor degree [5, 6] was hardly affected by variations of external pH [5]; in rat kidney, the rate of active methyl-α-D-glucoside transport showed an optimum around pH 7.5 [6].

An investigation of the possible relationship between the transport and metabolism of model sugars was also desirable in the light of observations from two laboratories that the renal transport of D-galactose [3] and 2-deoxy-D-glucose [4] was associated with a considerable accumulation of the respective sugar phosphates.

Preliminary data obtained in this investigation led to question quantitative values of free tissue sugars obtained by the then widely used technique first introduced by Cori and his associates [7–9], i.e. stopping metabolic processes by placing the tissue into a solution of $ZnSO_4$ and precipitating proteins and sugar phosphates by the addition of an equivalent amount of $Ba(OH)_2$ (see ref. 10). Observed discrepancies between values for free tissue sugar when employing several extraction and separation procedures could then be traced to the properties of the alkaline phosphatase in the tissue.

This communication presents an analysis of sources of possible errors in the determination of free tissue sugar in transport studies. The modified analytical procedure suggested here allows a rapid determination of both free and phosphorylated tissue sugars. Using this new method, several aspects of the relationship between the transport and metabolism of D-galactose are presented.

A preliminary communication of some of the results has been published [11].

METHODS

Renal cortex of healthy adult rabbits was used throughout.

Slices. Methods for the preparation and handling of slices were those currently used in this laboratory [1, 2] and are only summarized here. Slices of renal cortex (0.3–0.4 mm thick) were cut free-hand [12]. Pooled slices of one animal were kept in ice-cold balanced saline of the Krebs-Ringer type, buffered with a mixture of 0.308 M Tris and TES. Composition of the standard saline (mM): Na^+, 129; K^+, 6.6; Li^+, 6.6; Ca^{2+}, 2.8; Mg^{2+}, 1.3; Cl^-, 131; HCO_3^-, 4; acetate, 6.6; SO_4^{2-}, 1.3; $H_2PO_4^-$,

1.3; Tris/TES buffer mixture (usually pH 7.2), 13. A Na^+-free medium was prepared by an equivalent replacement of Na^+ by Li^+.

Incubation of the tissue was carried out in conical flasks (50 ml) containing 8 ml saline, using a metabolic shaker at usually 60–80 oscillations/min. The tissue was first preincubated aerobically (O_2) for 45 min at 25 °C in saline in order to achieve a steady state of tissue solutes, was then transferred into flasks containing 8 ml saline plus 1 mM labelled sugar and was again aerobically incubated. Unless otherwise stated, 60 min incubation was the standard procedure. The following activities were usually employed: ^{14}C, 0.02 μCi/ml; ^{3}H, 0.05–0.1 μCi/ml. After incubation, the blotted and weighed slices were taken for the determination of tissue sugar.

Substrate oxidation. The oxidation of D-[U-^{14}C]galactose to $^{14}CO_2$ was measured as described earlier [2]. The values are given in μmol galactose oxidized/g tissue dry weight.

Efflux experiments. The technique currently used in this laboratory [2] was employed. Tissue sugar (free and phosphorylated) was determined both at the start and the end of the wash-out procedure. Where values of tissue sugar at intermediate time intervals were required, separate runs of the wash-out were carried out, using slices from the same tissue pool. The efflux media were also analyzed for the presence of sugar phosphate, using the $ZnSO_4$ - $Ba(OH)_2$ separation procedure (see below). The results are plotted as the log % of activity originally present in the tissue after loading with the sugar, as a function of time. The justification of this procedure which relates the efflux of sugar to total tissue sugar, will be apparent from data given in Results.

Homogenates and tissue extracts. Homogenates of diced renal cortex were prepared and handled as described by Krebs [13] for liver. Membrane-free extracts of renal cortex were prepared (see ref. 14) by homogenizing diced tissue in four volumes of ice-cold medium containing 150 mM KCl, 1 mM Na_2-EDTA, 1 mM dithiothreitol, and 6 mM Tris/TES buffer mixture, pH 7.2, using a Polytron homogenizer (Brinkmann Instr.). The homogenate was centrifuged at 0 °C at 100 000 $\times g$ and the supernatant containing approx. 20 mg protein/ml, was employed.

Analytical methods

The method for the determination of free tissue sugar, as previously employed in this laboratory [15, 1, 2] was as follows: The tissue was placed into 3 ml of 0.05 M, $ZnSO_4$ which was assumed to stop all metabolic processes. After homogenization, 1 ml 0.15 M $Ba(OH)_2$ was added in order to precipitate proteins plus sugar phosphates [10], and in the clear supernatant free tissue sugar (see refs 9 and 5) was determined by scintillation spectrometry (Packard Instrument, Model 3320). Care was taken to balance the $ZnSO_4$ and $Ba(OH)_2$ solutions as described by Somogyi [10] in order to yield a neutral supernatant. Although the above technique differed slightly from that of Cori and associates [7–9] i.e. (a) the concentration of $ZnSO_4$ was 0.05 M, (not 0.19 M); and (b) some delay in the homogenization of the tissue occurred, these changes had been adopted after confirming that identical values were obtained for free tissue sugars by both procedures. As a result of the present investigation an improved analytical procedure will be described in Results.

Separation of free and phosphorylated sugar by ion-exchange chromatography. Neutral tissue extracts were employed. In the case of acidic extracts, e.g. tissue

extracted with ice-cold 5 % (w/v) trichloroacetic acid, (usually 4 ml), the deproteinizing agent was first removed by exhaustive extraction with ethyl ether. Three extractions with 4 ml ether were usually sufficient. A portion of the extract was then placed on a column (5 × 50 mm) of Dowex 1-X2 or Biorad AG1 × 2, 100–200 mesh, in the Cl-cycle, followed by twice 1 ml of water. Subsequent eluates with water contained only insignificant amounts of free sugar. Elution of the phosphorylated sugar carried out with twice 1 ml 2 M HCl, followed by twice 1 ml water. The recovery of counts was satisfactory, i.e. the sum of free and phosphorylated sugar equalled within limits of experimental error (101 ± 0.6 %, $n = 20$) the total counts placed on the column.

The results are expressed in μmol free and phosphorylated sugar per g tissue wet weight. The determination of the sugar concentration in the incubation medium at the end of the experiment (S_0) allows the calculation of the apparent intracellular concentration of the free or phosphorylated sugar, S_i, after correction for the extracellular (inulin, polyethylene glycol, or mannitol) space (see refs. 2 and 5). From these data, the apparent accumulation ratio, S_i/S_0, of the free sugar can be computed. Mean values ± S.E. are given.

Protein was determined by the method of Lowry et al. [16].

Paper chromatography. Tissue extracts were chromatographed by the descending technique using Whatmann No. 1 paper and *n*-propanol NH_4OH water (6 : 3 : 1, v/v) solvent mixture.

Student's *t*-test was used to assess the significance of the difference of mean values.

Materials. D-[1-^{14}C] Galactose, D-[U-^{14}C] galactose, D-[U-^{14}] galactose 1-phosphate, 2-deoxy-D-[1-^{14}C] glucose, methyl-α-D-[1-^{14}C] glucoside, and D-[1-^{14}C] mannitol were obtained from New England Nuclear Corp., Boston, Mass, D-[1-^{3}H] Galactose was purchased from Amersham-Searle Corp., Arlington Heights-Ill. All other reagents and materials were commercial products of the highest available purity.

RESULTS

The effect of Zn^{2+} on the determination of free tissue galactose

Preliminary experiments showed that using the trichloroacetic acid extraction procedure for total tissue sugar (see Methods) followed by a separation of free and phosphorylated sugar on an ion-exchange column, the values of free tissue sugar were less than 50 % of those found with the hitherto employed $ZnSO_4$ + Ba $(OH)_2$ method. An experiment was therefore designed to investigate whether this discrepancy could be produced by an incomplete stoppage of metabolic processes by the $ZnSO_4$ solution. Slices were first loaded with [^{14}C] galactose. Portions of the tissue were then treated as follows: (a) total tissue sugar was extracted with ice-cold 5 % (w/v) trichloroacetic acid, followed by the determination of free sugar after separation on the ion-exchange column; (b) tissue was placed into 3 ml boiling water for 10 min in order to stop enzymic reactions (see also ref 3) and free sugar was determined after deproteinization of the tissue homogenate with $ZnSO_4$ + Ba $(Ba(OH)_2$; and (c) free tissue galactose was analyzed using the hitherto employed $ZnSO_4$ + Ba $(OH)_2$ method after maintaining the slices for varying periods at room temperature in the $ZnSO_4$ solution.

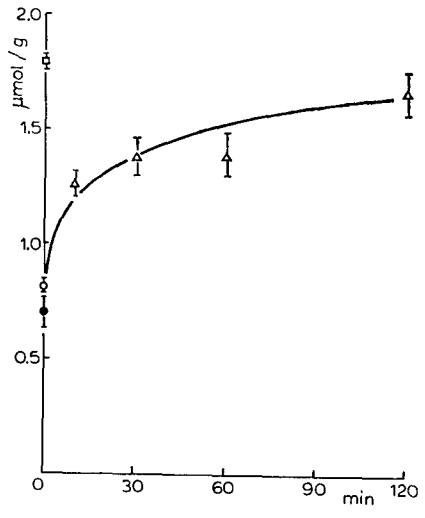


Fig. 1. Effect of Zn^{2+} on the determination of free tissue sugar. Groups of slices were loaded with [^{14}C]galactose by incubation for 60 min in saline containing 1 mM sugar (0.02 μCi/ml). Free tissue sugar was then determined in portions of the tissue after extraction of total sugar with boiling water and subsequent $ZnSO_4$ plus $Ba(OH)_2$ treatment (○), after varying periods in 0.05 M $ZnSO_4$ and subsequent deproteinization with $Ba(OH)_2$ (△), and after extraction with ice-cold trichloroacetic acid and determination of free (●) and phosphorylated (□) sugar by ion-exchange chromatography. Mean values, in μmol/g tissue wet weight ±S.E. ($n = 5$) are given.

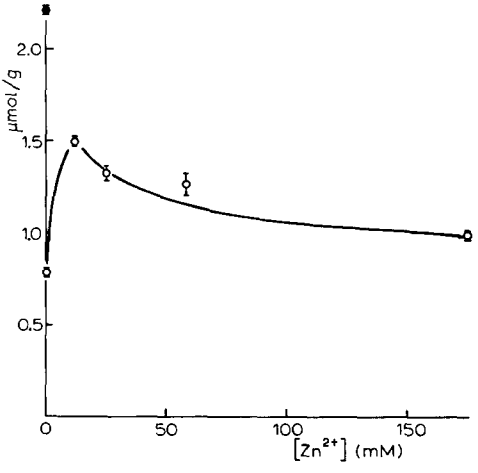


Fig. 2. Effect of varying [Zn^{2+}] on the determination of galactose in renal cortical slices. Slices were loaded with [^{14}C]galactose by aerobic incubation for 60 min in saline containing 1 mM sugar (0.02 μCi/ml). In a portion of the blotted and weighed slices free and phosphorylated galactose was determined after extraction of total sugar with boiling water and subsequent $ZnSO_4$ plus $Ba(OH)_2$ treatment. Further slices were placed for 30 min at room temperature into solutions of varying [Zn^{2+}]. After homogenization and addition of the appropriate amounts of $Ba(OH)_2$, free tissue sugar was determined in the supernatant. Values are given in μmol/g tissue wet weight ±S.E. ($n = 6$). Symbols: ○, free galactose; ●, galactose phosphate.

Fig. 1 shows that while the values for free sugar obtained by the procedures a and b were comparable, the amounts of free tissue galactose increased with prolonged contact of the slices with the $ZnSO_4$ solution. Such result suggested that at 20 °C a gradual conversion of sugar phosphate to free sugar took place in the $ZnSO_4$ solution. Fig. 2 shows that such conversion was greatest at low Zn^{2+} concentrations, but was still considerable even at 0.175 M $ZnSO_4$. Experiments not given here in detail were carried out to enquire whether a breakdown of galactose phosphate occurred also when the analytical conditions of Cori and associates [7–9] were strictly adhered to, i.e. the tissue was homogenized in 0.19 M $ZnSO_4$ and Ba $(OH)_2$ was added within 1 min after the slices had been placed into the homogenizing tubes. In the controls, where the tissue enzymes were inactivated at 100 °C (see above), the apparent accumulation ratio, S_i/S_0, for the free sugar was 2.3 ± 0.2 ($n = 6$). A value of 4.4 ± 0.1 was found after homogenizing the tissue in 0.19 M $ZnSO_4$, and this value is identical with that previously reported for the concentration gradient of free galactose established by the cells of rabbit renal cortex [9, 15, 1, 2]. It follows that contact of the tissue with Zn^{2+} prior to an inactivation of enzymes produces S_i/S_0 values of the order of 4, whereas low values (1.5–2.5) are found when the tissue enzymes are first inactivated either at 100 °C or by trichloroacetic acid (Fig. 1, and Table II below), implying that a breakdown of galactose phosphate takes place in the presence of Zn^{2+}.

Table I demonstrates that the dephosphorylation of galactose 1-phosphate added to a dilute homogenate of renal cortical tissue was increased by increasing pH (from 6.2 to 8.3). At each pH value Zn^{2+} proved to be a powerful activator. These results characterize the involved enzyme as a Zn^{2+}-activated alkaline phosphatase (phosphohydrolase, EC 3.1.3.1). Alkaline phosphatases isolated from the placenta [17] and from *Eccherichia coli* [18] have been described to be Zn-metalloenzymes.

TABLE I

EFFECT OF pH AND Zn^{2+} ON THE DEPHOSPHORYLATION OF GALACTOSE 1-PHOSPHATE BY A HOMOGENATE OF RENAL CORTEX

Tissue homogenate (see Methods) was diluted by the addition of 3.5 volume 0.15 M KCl and 0.5 volume 0.308 M Tris/TES buffer (pH 6.2, 7.2 and 8.2). The dilute homogenate (2 ml) was added to a mixture of 0.1 ml D-[^{14}C]galactose 1-phosphate (final concentration 1 mM, 0.02 μCi/ml) and 0.2 ml KCl or 0.2 ml $ZnSO_4$ (final concentration 17 mM). The assay mixtures were incubated aerobically (air) 30 min at 37 °C. The results are given in μmol free sugar formed per mg tissue per h using the $ZnSO_4$ plus $Ba(OH)_2$ analytical procedure.

pH (final)	Free galactose formed (μmol/g per h)	
	Without Zn^{2+}	17 mM Zn^{2+}
6.3	0.11	3.96
7.5	2.04	17.88
8.3	18.24	42.4

The determination of free and phosphorylated tissue sugar

Results given in Fig. 1 suggested the basis for the establishment of a simple technique for the determination of free and phosphorylated tissue sugar. The im-

TABLE II

COMPARISON OF TWO ANALYTICAL PROCEDURES FOR THE DETERMINATION OF FREE AND PHOSPHORYLATED SUGAR IN RENAL CORTICAL SLICES

Groups of slices (5–6 per flask) were incubated under standard conditions (O_2, 25 °C, 60 min) in salines containing one of the following sugars (1 mM): methyl-α-D-[^{14}C]glucoside; 2-deoxy-D-[^{14}C]-glucose; D-[^{14}C]galactose. For each sugar two flasks were used, and in the blotted and weighed tissue the sugars were determined by one of the two procedures: (a) Extraction of total sugar: 5 % ice-cold trichloroacetic acid; separation of free and phosphorylated sugar: ion-exchange chromatography. (b) Extraction: boiling water; free sugar was determined after precipitation of sugar phosphates with $ZnSO_4$ plus $Ba(OH)_2$, and the latter determined as the difference (total−free) sugar. Details of the experimental procedures are given in the text. Values are presented in μmol sugar/g tissue wet weight ±S.E.

Extraction procedure:		Trichloroacetic acid	Boiling water
Separation procedure:		Ion-exchange	$ZnSO_4$+$Ba(OH)_2$
Substrate	Fraction of tissue sugar	Tissue sugar (μmol/g)	Tissue sugar (μmol/g)
Methyl-α-D-glucoside	Total	1.74±0.08	1.72±0.13
	Free	1.71±0.07	1.67±0.13
	Phosphorylated	0.02±0.08	0.05±0.09
2-Deoxy-D-glucose	Total	2.08±0.05	2.03±0.07
	Free	0.75±0.03	0.77±0.03
	Phosphorylated	1.21±0.03	1.27±0.07
D-Galactose	Total	2.57±0.08	2.70±0.04
	Free	0.70±0.09	0.83±0.02
	Phosphorylated	1.72±0.04	1.87±0.06

proved analytical procedure prevents the breakdown of sugar phosphate in the course of the extraction of the tissue.

Table II summarizes the comparison of two analytical techniques indicated above, differing both in the method of extracting total tissue sugar and separation of free and phosphorylated sugar in the extract. Methyl-α-D-glucoside, D-galactose and 2-deoxy-D-glucose were used. In the first method, total sugar was extracted from the slices with boiling water and free sugar was determined after precipitation of proteins and sugar phosphates by the $ZnSO_4$+Ba $(OH)_2$ technique. The second procedure consisted of extracting total sugar with ice-cold 5 % trichloroacetic acid and separation of free and phosphorylated sugar by ion-exchange chromatography. It will be noticed that within the limits of experimental error both techniques yielded identical data for total, free and phosphorylated sugar. Results not given here in detail showed that 50 % aqueous ethanol at 80 °C [19] or ice-cold 5 % $HClO_4$ served equally well for the extraction of total tissue sugar.

The advantages of the extraction procedure employing water 100 °C and the $ZnSO_4$+Ba $(OH)_2$ technique for the determination of free tissue sugar in the supernatant are as follows: (a) the method is simpler than all other techniques tested; and (b) a neutral tissue extract is obtained. It will be shown elsewhere [20] that C_1-phosphate esters of 2-deoxy-sugars are acid labile and are broken down even in ice-cold trichloroacetic acid or $HClO_4$ solutions. It should be noted, however, that by this method values for sugar phosphates are obtained only indirectly, i.e. as the difference

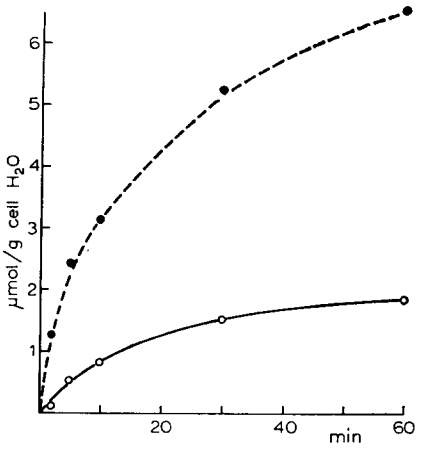


Fig. 3. Uptake of D-galactose by renal cortical cells. Tissue was incubated aerobically (O_2) at 25 °C in Na^+-free (Li^+) saline containing 1 mM [^{14}C]galactose (0.02 µCi/ml). Free (○) and phosphorylated (●) sugar were determined by trichloroacetic acid extraction and ion-exchange separation procedure. Each point is the mean of two analyses. Values are given in µmol sugar/g cell water.

between total and free tissue sugar. Where emphasis is placed on the determination of sugar phosphates, the separation technique by ion-exchange chromatography is preferable.

The uptake of D-*galactose by tissue slices*

Time-course experiments on the uptake of D-galactose by slices revealed that galactose phosphate appeared in the tissue within the first few minutes of incubation. In order to ascertain the time-course of the appearance of both free and phosphorylated sugar in the cells it was first established that D-mannitol filled the extracellular (inulin) space within 2 min of incubation at 25 °C (details not given here); thus, a correction for the extracellular space even at such short time period is justified. The error introduced by this procedure owing to a slight entry of mannitol into the cells [5] is insignificant. Fig. 3 gives the result of one such experiment. It will be seen that at any time period the apparent intracellular concentration of galactose phosphate greatly exceeded that of the free sugar. The cellular level of the free sugar reached a steady state within 60 min incubation, and the mean apparent accumulation ratio, S_i/S_0, was 1.45 ± 0.09 (range 1.2–2.5. 17 animals). S_i/S_0 values of 1.2–2.5 were obtained with standard salines as well as with Na^+-free (Li^+) media. It should be noted that the cells continued to accumulate galactose phosphate even after a steady state of free sugar has been reached. This fact may account for considerable variations in the values for tissue galactose phosphate observed in various experiments.

Paper chromatography revealed that under the given experimental conditions little glucose was formed from galactose.

The fact that the appearance of galactose phosphate preceded that of the free sugar in the cells raised questions as to the first step in the transport process. An attempt was made to clarify this point as follows (see, e.g. ref. 21): Slices were first

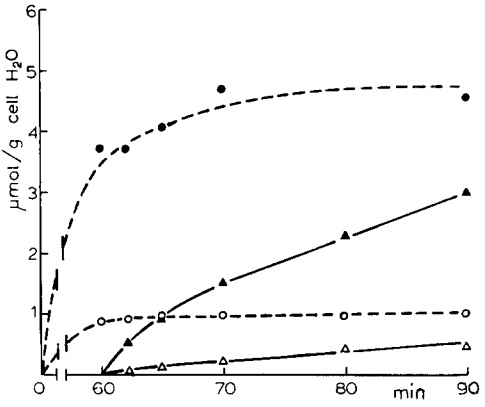


Fig. 4. The exchange of ^{14}[C]galactose with cellular pools of free and phosphorylated sugar. For details of the experiment, see text. ○, ●, [^{3}H]galactose; △, ▲, [^{14}C]galactose; open symbols, free sugar; full symbols, galactose phosphate. Tissue sugar was determined by the trichloroacetic acid extraction and ion-exchange separation procedure. Each point (in µmol/g cell water) is the mean of three analyses.

incubated for 60 min in saline containing 1 mM [^{3}H]galactose (0.1 µCi/ml) and were then transferred for varying time periods to identical saline also labelled with 0.02 µCi/ml [^{14}C]galactose. The time course of the entry of both labels into the cellular pool of free and phosphorylated sugar was followed, after correction for the extracellular space. As pointed out above, it was justified to compute the apparent intracellular concentrations of free and phosphorylated sugar in view of the rapid filling of the extracellular space by mannitol, a marker of a molecular weight comparable to that of galactose. The results given in Fig. 4 show that [^{14}C]galactose appeared in the cellular pool of galactose phosphate considerably faster than in that for the free sugar. The simplest explanation for such a result is the assumption that phosphorylation of galactose represents a step in the transfer of this sugar across the cell membrane. Such a view has also been put forward for the transport of the sugar across the antiluminal face of the flounder renal tubular cells on the basis of the effects of phlorizin and its derivatives on the appearance of free and phosphorylated galactose in the cells [22].

The effect of pH on the transport and phosphorylation of D-*galactose in renal cells*

A reexamination of the pH effect on the transport of D-galactose in renal cortical slices (Fig. 5) showed that increasing saline pH affected the steady-state cellular levels of free sugar only to a minor degree whereas that of galactose phosphate linearly increased. Since variations of external pH also produce concomitant changes in the apparent intracellular pH [1], the effect of pH on the activity of a crude renal galactokinase preparation was studied. The results, given in Fig. 6, demonstrate that the phosphorylation of D-galactose increases with pH increasing from 5.6 to 7.8 (final values). The activity of liver galactokinase shows a sharp optimum at pH 8 [23]. The data suggest that external pH may act on the transport process indirectly, i.e. by activating the intracellular phosphorylation of galactose; an additional, more direct effect on the actual transport step cannot, however, be excluded.

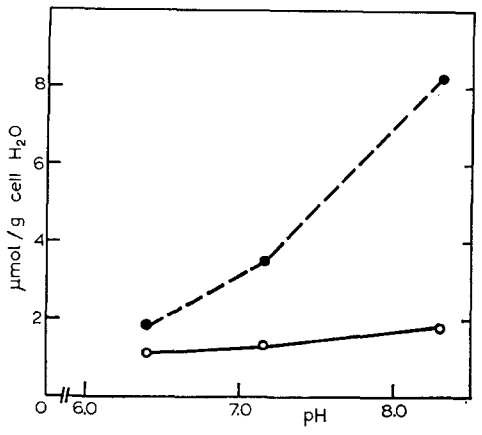


Fig. 5. The effect of pH on the accumulation of D-galactose in renal cortical slices. Groups of slices (4 per flask) were first preincubated 45 min aerobically (O_2) at 25 °C in salines of varying pH: Initial values: pH 6.18; 7.18 and 8.25. The pH was varied using appropriate mixtures of 0.308 M Tris and TES; final concentration of the buffer mixture: 15 mM. The tissue was then incubated 60 min under identical conditions in salines containing 1 mM [^{14}C]galactose (0.02 µCi/ml). Mean values of free (○) and phosphorylated (●) tissue sugar (in µmol/g cell water) and final pH values are given.

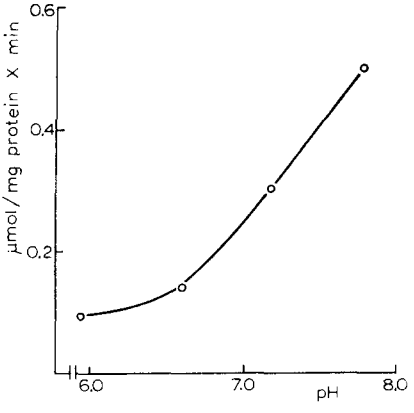


Fig. 6. The effect of pH on the phosphorylation of galactose in a cell-free extract of renal cortex. Components (final concentrations) of the assay mixture (2 ml): 0.15 M KCl, 1 mM $MgCl_2$, 1 mM Na_2-EDTA, 1 mM dithiothreitol, 2.5 mM ATP, 2.5 mM D-[^{14}C]galactose (0.1 µCi/ml), 30 mM Tris/TES buffer mixture of appropriate pH, tissue extract (10 mg protein). Incubation: 10 min, 37 °C. Abscissa: final pH values; ordinate: µmol sugar phosphate formed · (mg protein · min)$^{-1}$.

The effect of phlorizin and phloretin on the uptake and metabolism of D-*galactose in renal cortical cells*

Previous data [23] have indicated that D-galactose is transported into renal cortical cells by two parallel pathways, only one of which is Na^+ independent. The carrier for the Na^+-dependent transport pathway appears to have a high affinity for phlorizin whereas the Na^+-independent transport system was shown to be phloretin sensitive (see ref. 22). Experiments were carried out to test the effects of both phlorizin

and phloretin (0.5 mM) on the transport and metabolism of D-galactose. Two experimental conditions were used: (1) The tissue was incubated in a medium containing galactose without (control, flasks a) and with the respective inhibitors (flasks b). (2) The slices were preincubated in salines containing only the respective inhibitors (0.5 mM). Subsequently, the tissue was briefly washed in fresh saline in order to remove adhering or weakly bound inhibitor, and was then incubated in saline containing only the labelled sugar (flasks c). Data to be presented elsewhere showed that under the conditions of preincubation particularly phlorizin interacted with high affinity sites in the tissue, and subsequent incubation with model sugars such as methyl-α-D-glucoside showed complete inhibition of the active transport system.

TABLE III

EFFECT OF PHLORIZIN ON THE UPTAKE OF D-GALACTOSE BY RENAL CORTICAL SLICES

Groups of slices (6 per flask) were preincubated (O_2, 25 °C) for 45 min in standard (Na^+) or Na^+-free (Li^+) salines without (flasks a and b) or with 0.5 mM phlorizin (flasks c). Subsequently, the tissue was transferred into flasks containing 1 mM D-[^{14}C]galactose (0.02 μCi/ml) and incubated (O_2, 25°, 60 min). Transfers: from flasks a: direct (controls); from flasks b: transfer after 2 min wash in fresh saline; from flasks c: transfer into saline also containing the inhibitor. Mean values of free and phosphorylated sugars are given in μmol/g tissue wt weight, ±S.E.

Flask	Fraction of tissue sugar	Tissue sugar (μmol/g)	
		Na^+-saline	Li^+-saline
a (control)	Free	0.89±0.04	0.74±0.04
	Phosphorylated	1.66±0.04	0.79±0.05
b (incubation with phlorizin)	Free	0.69±0.02	0.59±0.01
	Phosphorylated	1.11±0.05	0.60±0.03
c (preincubation with phlorizin)	Free	0.77±0.02	0.65±0.01
	Phosphorylated	1.30±0.03	0.58±0.01

An inspection of data in Table III shows that, as compared with the control, phlorizin inhibited the tissue levels of both free and phosphorylated galactose. The effect of phlorizin was marked in the presence of Na^+, and could in part be reversed by washing out (i.e. the preincubation procedure, flask c). Such result indicates that a portion of the galactose transport has been mediated by a carrier with a relatively low affinity for phlorizin.

Evidence will be presented elsewhere to show that the inhibitory effect of phlorizin on the uptake of galactose is not produced by an interference with the energy metabolism of the cells: Under the above experimental conditions phlorizin did not affect the cellular levels of ATP, ADP and AMP, and its inhibitory effect on tissue respiration and oxidation of metabolic substrate (acetate) was less than 10 %.

Data given in Table IV demonstrate that 0.5 mM phlorizin inhibited only by about 10 % the oxidation of D-[U-^{14}C]galactose to $^{14}CO_2$. A more marked inhibition (33 %, $P < 0.005$) of galactose oxidation was produced by the absence of external Na^+. These results, taken in conjunction with those given in Table III, indicate that it is particularly the Na^+-independent transport system by which D-galactose enters

TABLE IV

EFFECTS OF Na^+ AND PHLORIZIN ON THE OXIDATION OF D-GALACTOSE BY RENAL CORTICAL TISSUE

Slices (2 per flask) were first incubated 45 min (25 °C), O_2 in Na^+- or Na^+-free (Li^+-) salines. The tissue was then transferred into identical salines containing 1 mM D-[U-^{14}C]galactose (0.05 μCi/ml) without (control) and with inhibitor. The stoppered flasks were equipped with a center well containing 0.1 ml 1 M NaOH and a wick of filter paper. After incubation (90 min, 25 °C, O_2) the flasks were acidified with HCl and incubated 50 min in order to complete the absorption of retained $^{14}CO_2$ by NaOH. The contents of the center well were quantitatively transferred to scintillation vials for the assay of $^{14}CO_2$. The dry weight of the tissue in each flask was also determined. The values are given in μmol galactose oxidized by 1 g tissue weight dry per h ±S.E. ($n = 6$).

Exp. No.	Saline	Inhibitor	Galactose oxidized (μmol/g per h)
1	Na^+	None (control)	1.78 ± 0.07
	Na^+	Phlorizin, 0.5 mM	1.61 ± 0.03
2	Na^+	None (control)	1.35 ± 0.05
	Li^+	None	0.94 ± 0.02

the cells and serves as substrate in the metabolic pool. However, as opposed to the behavior of D-glucose in renal and intestinal epithelial cells, (unpublished data), D-galactose entering the cells by the Na^+-dependent, phlorizin-sensitive transport pathway also in part mixes with the cellular metabolic pool and is oxidized to CO_2.

A study of the effect of 0.05–0.5 mM phloretin on the uptake and cellular oxidation of D-galactose revealed that this compound is a more potent inhibitor than phlorizin. However, such effects of phloretin may lack specificity since this compound also inhibits tissue respiration and the oxidation of such tissue substrates as acetate (unpublished data).

The efflux of D-*galactose from renal cortical slices*

Another facet of the relationship between transport and metabolism of sugars in renal cells was revealed by a reinvestigation (see ref. 2) of the efflux of galactose from slices. Data for such experiments with D-galactose are given in Fig. 7 and Table V. In these experiments the tissue was first loaded with [^{14}C]galactose by preincubation for 60 min under standard experimental conditions. One batch of slices was then used for the determination of the initial content of total, free and phosphorylated sugar. Another batch was employed for the actual wash-out curve and the final content of tissue sugar was also determined. Under identical conditions, tissue sugar was also determined at two intermediate time intervals. The efflux curve was expressed in the usual fashion, by plotting the log % of the activity remaining in the tissue (i.e. total tissue sugar) against time.

Fig. 7 shows that the efflux curve, related to total tissue sugar, also described the changes occurring with both free and phosphorylated tissue galactose. If the efflux of galactose occurred only from the cellular pool of free sugar, the tissue level of galactose phosphate would have remained constant, i.e. at log % = 2.0. Since within the limits of experimental error only free sugar was washed out from the tissue, the above result suggested that a portion of the free galactose appearing in the efflux media

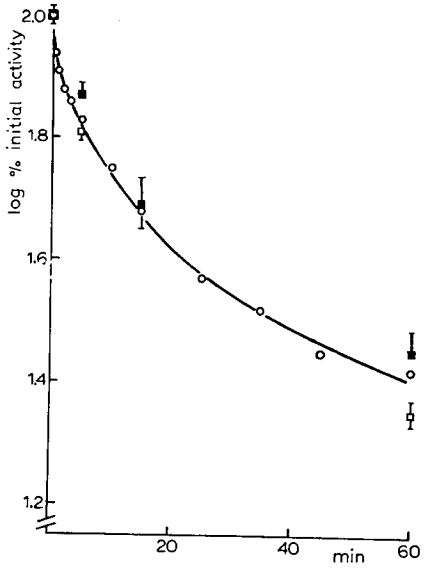


Fig. 7. Efflux of galactose from renal cortex slices and concurrent changes in the tissue level of free and phosphorylated sugar. Groups of slices were first loaded with [^{14}C]galactose by aerobic incubation in saline containing 1 mM sugar (0.1 μCi/ml). A portion of the slices was then taken for the determination of the initial activity corresponding to total, free and phosphorylated sugar. Further slices were blotted and were placed into three washout tubes and the efflux of galactose into series of tubes (each containing 10 ml sugar-free saline) was followed for varying time periods; the final values of free and phosphorylated tissue sugar were then determined. Ordinate: log % of initial values in the tissue; ○, total activity from wash-out curve; □, tissue free sugar; ■, tissue galactose phosphate. Mean values of tissue sugar ±S.E. (n = 5) are given.

TABLE V

BALANCE SHEET OF TISSUE GALACTOSE DURING AN EFFLUX EXPERIMENT

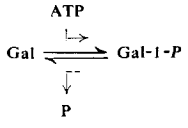
16 slices of renal cortex were loaded with D-[^{14}C]galactose by 60 min incubation (O_2 as gaseous phase) at 25 °C in saline containing 1 mM sugar (0.1 μCi/ml). Initial tissue sugar was then determined in eight slices. The remaining tissue was used to follow the wash-out of the sugar at 25 °C into a series of tubes each containing 10 ml sugar-free saline. Total, free and phosphorylated galactose were then determined in the tissue and in the wash-out media. Normalized data, in μmol/g tissue wet weight ±S.E. are given.

	[^{14}C]galactose (μmol/g)		
	Total	Free	Phosphorylated
Tissue: initial	2.78±0.04	0.78±0.02	2.00±0.03
Tissue: final	0.44±0.01	0.11±0.01	0.33±0.02
Efflux media	2.53	2.34	0.19

was derived from the pool of galactose phosphate. An inspection of data given in Table V, obtained in another such experiment clarifies this point further. Here, 2.34 μmol free galactose was washed out from 1 g tissue in 90 min. This amount exceeded by a factor of 3 that initially present in the tissue (0.78 μmol). At the same time, the

tissue content of galactose phosphate decreased by 1.67 μmol. The recovery of sugar in this experiment was satisfactory for such procedures: In the efflux media, 2.53 μmol galactose was found, and 0.44 μmol were recovered in the tissue. The sum, i.e. 2.97 μmol, represent 106 % of the initial value of 2.78 μmol total tissue sugar.

In the light of data given above, it may be assumed that the intracellular levels of free and phosphorylated galactose are at a steady state which is in part governed by the activity of two enzymes, i.e. a kinase and a Zn^{2+}-activated (alkaline) phosphatase



In such a system, the wash-out of galactose from the tissue would produce a dephosphorylation of galactose phosphate.

Indications that the same set of processes is operative for the relationship of free and phosphorylated 2-deoxy-D-glucose have been presented previously for the flounder renal tubular cells. On the other hand, Elsas and Macdonell [4] did not observe a dephosphorylation of 2-deoxy-D-glucose 6-phosphate in slices of hamster kidney cortex, and we confirmed their observations also for the rabbit kidney (details not given here).

DISCUSSION

A comparison of data obtained by the previous and improved analytical procedures for the determination of free tissue sugar is of interest. At 1 mM galactose, the concentration gradient of free sugar established by rabbit renal cortical cells, i.e. $S_i/S_o = 1.45$, is considerably lower than that reported earlier from several laboratories [9, 14, 2], i.e. about 4.0. The revised value is close to that reported by McNamara and Segal [3] for the rat renal cortex. Present experience in this laboratory shows that the elimination of analytical error due to the activity of the Zn^{2+}-activated phosphatase in the tissue necessitates a lowering of previously reported values for the steady-state accumulation ratio which renal cortical cells establish by the active transport of metabolizable sugars, e.g. 2-deoxy-D-galactose [20], 2-deoxy-D-glucose and D-mannose [24]. However, qualitative conclusions as to the nature and properties of the transport process were not found to be affected by such revision. In particular, Fig. 3 shows that the transport of D-galactose against its concentration gradient takes place also in the absence of external Na^+ (see ref. 25), although in any experiment the total amount of sugar taken up by the tissue tended to be increased by the presence of Na^+ (see, e.g. Table III).

The complex relationship between the transport and phosphorylation of D-galactose in renal cells requires a closer analysis. Kinetically, the appearance of cellular galactose phosphate preceded that of free sugar under conditions of both net uptake (Fig. 3) and steady-state exchange (Fig. 4). This observation is difficult to reconcile with the generally held view that the active transport mechanism brings about an up-hill flow of the free sugar across the membrane with a subsequent intracellular phosphorylation. The alternative possibility, i.e. the phosphorylation of galactose representing a step in a transfer of the sugar across the cell membrane

cannot at present be excluded. A study of the effect of phlorizin and its derivatives on the equilibrating transport system of D-galactose and 2-deoxy-D-galactose at the antiluminal face of the renal tubular cells of the flounder led to similar tentative conclusions [22].

Data on the effect of pH on the galactokinase and on the transport system shared by galactose and its 2-deoxy derivative suggest that galactokinase is not involved in the actual transfer process at the membrane. Whereas increasing pH stimulated the galactokinase-catalyzed phosphorylation of both substrates (Fig. 6 above, and Table II in ref. 20), opposite effects on the uptake of both sugars were found in slices, i.e. an increased uptake of galactose (Fig. 5) and a depression of the transport of 2-deoxy-D-galactose (Fig. 3 in ref. 20). Further investigation of the specificity of transport and phosphorylation using membranes of renal cortical cells is desirable.

D-Galactose is reabsorbed from the lumen of the renal tubule [26–28]. There is ample evidence to show that this Na^+-dependent, active reabsorptive mechanism is shared with D-glucose, is localized at the brush border of the proximal tubular cells [29–31] and is highly sensitive to inhibition by phlorizin [26, 28, 29]. On the other hand, the Na^+-independent transport system [1, 23, 25] investigated in the present report in more detail (Fig. 3, Tables III and IV) appears to be localized at the antiluminal cellular face. This suggestion arises from the following set of information on the relative specificities of galactose transport in renal cells: (a) In vivo, D-galactose interacts not only with the brush border but also with the antiluminal membranes of renal cells [27]. (b) In micropuncture studies in rats, 2-deoxy-D-galactose was not reabsorbed at sites actively transporting galactose (and glucose) [29]. (c) In vitro, using slices of rabbit renal cortex and teased tubules of the flounder, two mechanisms of D-galactose transport could be distinguished, differing in their Na^+ requirement as well as in their sensitivity to phlorizin and phloretin. The carrier involved in the Na^+-independent transport system is shared by D-galactose and 2-deoxy-D-galactose, but not by D-glucose [20, 22] and its affinity for phloretin is higher than for phlorizin [23, 22]. In the tubular cells of rabbit kidney, this transport system is capable of establishing a modest concentration gradient of free sugar (Fig. 3). In the flounder renal tubule this transport pathway is localized at the antiluminal cellular face but does not appear to transport the substrates against their concentration gradients [22].

The above view is consistent with the recent report that D-galactose can be transported against a small concentration gradient by a Na^+-independent mechanism

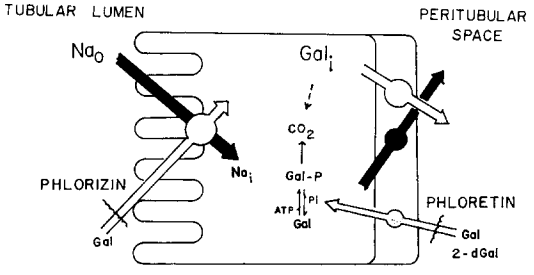


Fig. 8. Tentative model for the transport and metabolism of D-galactose in cells of the proximal renal tubule.

localized at the antiluminal face of intestinal mucosal cells [31]. Transport system(s) for monosaccharides with the above localization appear to be characterized by a high sensitivity to phloretin, in contrast to a relatively low sensitivity to phlorizin [32].

At the present level of knowledge the following tentative model of the relationship between transport and metabolism of D-galactose in renal tubular cells can be suggested (Fig. 8): (1) At the luminal face, D-galactose is transported into the cells against its concentration gradient by a mechanism coupled to the down-hill flux of Na^+; the transport site is shared by D-glucose, methyl-α-D-glucoside and both anomers of methyl-D-galactoside [29, 33] and has a high affinity for phlorizin. Galactose entering the cells by this transport pathway mixes with the metabolic cellular pool only to a minor extent as evidenced by the marginal inhibition of galactose oxidation by phlorizin and the relatively small effect produced by the absence of Na^+ on the oxidation of galactose (Table IV). (2) At the antiluminal face, D-galactose is transferred into the cells, possibly by a phosphotransferase system. This transport pathway, shared with 2-deoxy-D-galactose, is Na^+ independent, appears to phloretin sensitive and displays only a low affinity for phlorizin. It is this transport pathway by which D-galactose predominantly enters the cellular metabolic pool, as indicated by the marked inhibition of galactose oxidation by phloretin (Table IV). (3) The intracellular levels of free and phosphorylated galactose are governed not only by the respective transport systems (including the efflux mechanism) but also by the activities of galactokinase and a Zn^{2+}-activated alkaline phosphatase; the first of these enzymes is part of the enzymic system feeding cellular galactose to the Leloir metabolic pathway (see ref. 34) and hence to oxidation of the sugar.

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